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Review

Toll-like receptors in bony fish: From genomics to function

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ABSTRACT

Receptors that recognize conserved pathogen molecules are the first line of cellular innate immunity defense. Toll-like receptors (TLRs) are the best understood of the innate immune receptors that detect infections in mammals. Key features of the fish TLRs and the factors involved in their signaling cascade have high structural similarity to the mammalian TLR system. However, the fish TLRs also exhibit very distinct features and large diversity which is likely derived from their diverse evolutionary history and the distinct environments that they occupy. Six non-mammalian TLRs were identified in fish. TLR14 shares sequence and structural similarity with TLR1 and 2, and the other five (TLR19, 20, 21, 22 and 23) form a cluster of novel TLRs. TLR4 was lost from the genomes of most fishes, and the TLR4 genes found in zebrafish do not recognize the mammalian agonist LPS and are likely paralogous and not orthologous to mammalian TLR4 genes. TLR6 and 10 are also absent from all fish genomes sequenced to date. Of the at least 16 TLR types identified in fish, direct evidence of ligand specificity has only been shown for TLR2, TLR3, TLR5M, TLR5S and TLR22. The common carp TLR2 was shown to recognize the synthetic triacylated lipopeptide Pam_3CSK_4 and lipopeptides from gram positive bacteria. The membrane-bound TLR5 (TLR5M) signaling in response to flagellin in rainbow trout is amplified through interaction with the soluble form (TLR5S) in a positive loop feedback. In Fugu, TLR3 is localized to the endoplasmic reticulum (ER) and recognizes relatively short dsRNA, while TLR22 has a surveillance function like the human cell-surface TLR3. Genome and gene duplications have been major contributors to the teleost's rich evolutionary history and genomic diversity. Duplicate or multi-copy TLR genes were identified for TLR3 and 7 in common carp, TLR4b, 5, 8 and 20 in zebrafish, TLR8a in rainbow trout and TLR22 in rainbow trout and Atlantic salmon. The main task for current and near-future fish TLRs research is to develop specificity assays to identify the ligands of all fish TLRs, which will advance comparative immunology research and will contribute to our understanding of disease resistance mechanisms in fish and the development of new adjuvants and/or more effective vaccines and therapeutics.

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Abbreviations: CD14, cluster of differentiation 14; ER, endoplasmic reticulum; IL, interleukin; IL1R, interleukin-1 receptor; IRAK, IL1R-associated kinase; IRF, interferon regulatory factor; LBP, LPS-binding protein; LPS, lipopolysaccharide; LRR, leucine-rich repeats; MD-2, myeloid differentiation protein-2; MyD88, myeloid differentiation primary response gene/protein 88; NF-kB, nuclear factor kB; PAMP, pathogen associated molecular pattern; PRR, pattern recognition receptor; TICAM, TIR-containing adaptor molecule; TIR, Toll/interleukin-1 receptor resistance domain; TLR, Toll-like receptor; TRAF6, tumor necrosis factor receptor-associated factor 6; TRIF, TIR domain-containing adaptor inducing interferon.

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1. Foreground

This review is not the only one on Toll-like receptors in fish which has recently been published. As the recent reviews by Rebl et al. (2010) and Takano et al. (2010) provided comprehensive literature surveys and detailed descriptions of the known similarities and differences between the fish and mammalian TLR signaling systems, I chose to focus on the genomics perspective of TLRs research in fish with two primary objectives: (i) To remind the reader of the various genome duplications during vertebrate evolution and relating that to the evolution of the TLR-signaling cascade in fish; and (ii) to provide a comprehensive literature guide on teleost TLR ligands.

2. Introduction—mammalian TLRs and their known ligands

Receptors that recognize conserved pathogen molecules are part of the ancient innate arm of the immune system and are conserved in both invertebrate and vertebrate lineages. The family of Toll-like receptors (TLRs) is the best understood of the innate immune receptors that detect infections. TLRs are transmembrane proteins that recognize conserved pathogen structures to induce immune effector molecules. In vertebrates, TLRs can distinguish among classes of pathogens and serve an important role in orchestrating the appropriate adaptive immune responses (Takeda et al., 2003). TLRs contain an extracellular N-terminus with leucine-rich repeat region (LRR), a transmembrane domain and an intracellular C-terminus with a Toll/IL-1 receptor domain (TIR). An illustration of the TLRs domains is presented in a schematic diagram of several fish TLRs (Fig. 1). The cytoplasmatic TIR domain harbors conserved amino acids that have been shown to be involved in the signaling as well as in the localization of the TLR (Slack et al., 2000; Funami et al., 2004), while the LRR region is involved in pathogen recognition (Bell et al., 2003). In humans, 10 TLRs have been described and shown to identify distinct pathogen associated molecular patterns (PAMPs), which are molecules characteristic of a class of pathogens and are essential for pathogens survival (Iwasaki and Medzhitov, 2004; Pasare and Medzhitov, 2005; Roach et al., 2005; Temperley et al., 2008). Overall, 21 distinct TLR gene types have been identified to date from various animal species (Roach et al., 2005; Temperley et al., 2008).

TLRs recognize their ligands through interactions with the LRRs and trigger the activation of intracellular signaling through a cytoplasmic myeloid differentiation primary response protein 88 (MyD88)-dependent pathway or a MyD88-independent pathway. All mammalian TLRs, with the exception of TLR3, depend at least in part on the MyD88 adaptor for full signal transduction activity. In the MyD88-dependent pathway, MyD88 recruits the interleukin-1 receptor-associated kinases (IRAKs) and TNF receptor-associated factor 6 (TRAF6), which in turn activate downstream genes in TLR signal transduction. Ultimately, through the activation of NF-κB, IRF3 or IRF7, the TLR signaling pathways induce production of proinflammatory cytokines including interleukin (IL), tumor necrosis factor (TNF), and type I interferon (IFN) molecules that mediate direct defense responses and alert adaptive immune cells to the presence of a pathogen (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2010). In recent years the TLR signaling cascade has been intensively studied in mammals. The discovery of several TIR-containing adaptors led to the current understanding that individual TLR types recruit distinct cytosolic adaptors which can trigger specific responses to the infecting microbes (Kawai and Akira, 2010; Akira et al., 2006). In addition, cell-type specific signaling defined by its immunological properties can alter the response triggered by the same TLR signal in different cell types (Barbalat et al., 2009).

Two major TLR subfamilies were identified in human. TLR1, 2, 4, 5, 6 and 10 are the members of the cell surface sub-family recognizing microbial lipids, sugars and proteomes (Hajjar et al., 2001; Hayashi et al., 2001; Hoshino et al., 1999; Takeuchi et al., 2001, 2002; Underhill et al., 1999; Werts et al., 2001). TLR3, 7, 8 and 9 are the members of the nucleic acid-sensing subgroup recognizing nucleotide derivatives of viral or bacterial origin (Diebold et al., 2004; Latz et al., 2004; Gorden et al., 2005; Alexopoulou et al., 2001; Gibbard et al., 2006). The nucleic acid TLRs are localized in various intracellular compartments. Three TLR genes found in mice, TLR11, 12 and 13, have been lost from the human genome, and of the three, only one ligand for TLR11 has been identified to date (Beutler, 2009; Yarovinsky et al., 2005). The ligand is Profilin, a protein from a protozoan parasite, suggesting that TLR11 is also a cell surface receptor.

The TLR1, 2, 6 and 10 genes form a phylogenetically related cluster based on sequence similarities and genomic structures (Roach et al., 2005; Temperley et al., 2008), and in their dimeric combinations they cover broad variations of bacterial peptidoglycans and lipoproteins (Medzhitov, 2001). They are primarily located on the cell surface and upon activation they induce NF-κB expression through the recruitment of IL-1R signaling molecules. (Medzhitov et al., 1998; Shimizu et al., 2005, 2007). In mammals, the synthetic diacylated (Pam_2CSK_4) and triacylated (Pam_3CSK_4) lipoproteins are known experimental agonists of TLR2/6 and 2/1 heterodimers (Takeuchi et al., 2002; Shimizu et al., 2005, 2007). In addition, TLR2 has been implicated in the recognition of zymosan from fungi, tGPI-mucin from *Trypanosoma cruzi* and the hemagglutinin protein from measles virus (Akira et al., 2006).

The mammalian TLR4 is a central protein in the receptors complex responding to bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. The transfer of monomeric LPS to TLR4 on the cell surface is mediated through a complex of LPS-binding protein (LBP) and cluster of differentiation 14 (CD14) (Schumann et al., 1990; Wright et al., 1990). TLR4 forms a receptor complex with myeloid differentiation protein 2 (MD2), and together they function as the main cell-surface LPS-binding component (Hoshino et al., 1999; Rhee and Hwang, 2000). In addition to binding LPS, TLR4 has been implicated in the recognition of viral surface proteins (Akira et al., 2006).

TLR5 in mammals and fish has been shown to recognize the flagellin protein component of bacterial flagella and be responsible for flagellin-mediated NF-κB activation (Hayashi et al., 2001; Tsujita et al., 2004). In mammals it has also been implicated to be involved in adaptive immunity through promoting of the differentiation of helper T cells and naïve B cells into immunoglobulin A – producing plasma cells in response to flagellin (Uematsu et al., 2008).

In mammals, TLR3 has been shown to respond to double-stranded RNA (dsRNA), TLR9 to unmethylated CpG DNA and TLR7 and TLR8 were shown to be activated by synthetic antiviral imidazoquinoline compounds and were implicated in recognizing single-stranded RNA (Diebold et al., 2004; Latz et al., 2004; Gorden et al., 2005; Alexopoulou et al., 2001; Gibbard et al., 2006). These TLRs are primarily located in the endoplasmic reticulum and in lysosomal-like vesicles and are thought to have an important role

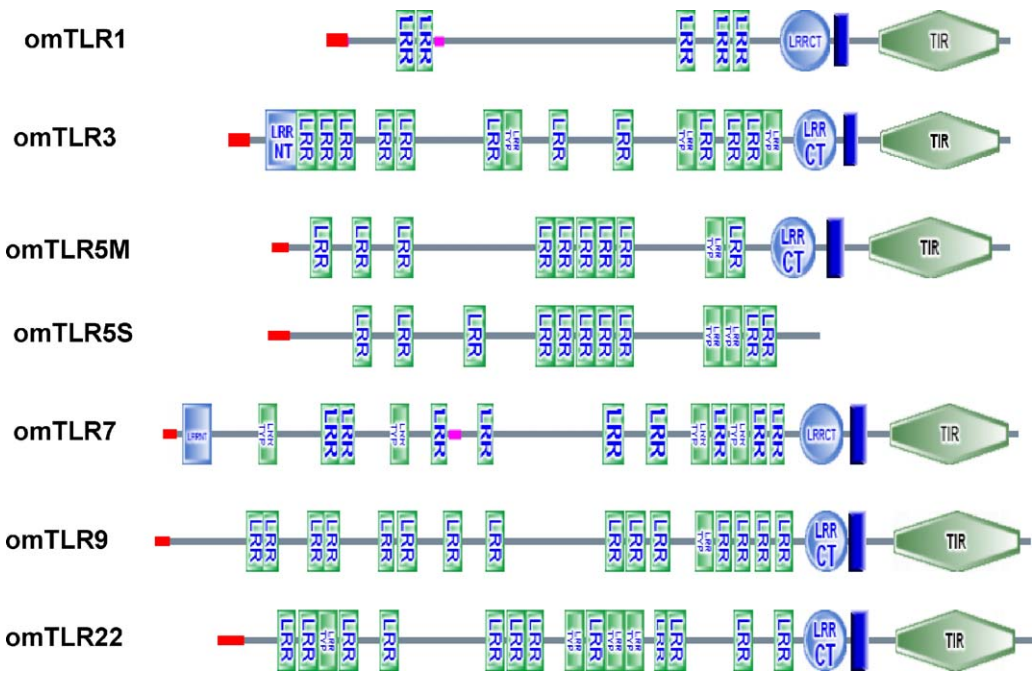

Fig. 1. Schematic domain organization of the rainbow trout TLR1, 3, 5M, 5S, 7, 9 and 22. The domain organization of each TLR was predicted from the amino acid sequences using the SMART, TMHMM and SignalP programs. LRR: leucine-rich repeat. TIR: Toll/IL-1 receptor. NT: N- (amino) terminal. CT: C- (carboxyl) terminal: ( ) Transmembrane domain; ( ) Low compositional complexity segment; ( ) Signal peptide.

in antiviral immunity (Barton and Kagan, 2009). The TLR7, 8 and 9 genes form a phylogenetically related cluster based on sequence similarities and genomic structures (Roach et al., 2005; Temperley et al., 2008). Upon activation they recruit MyD88 that through several effector molecules initiates the activation of two major signaling pathways resulting in the production of pro-inflammatory cytokines through the activation of NF-κB and/or type I interferons through the activation of IRF7 (Takeda et al., 2003; Negishi et al., 2006). TLR3 activation leads to cytokine secretion, especially type I interferon-beta (Alexopoulou et al., 2001). The TLR3 signal transduction is through the TRIF dependent pathway resulting in the production of pro-inflammatory cytokines through the activation of NF-κB and type I interferons through the activation of IRF3 (Kawai and Akira, 2010). Although studies suggest that TLR3 is not universally required for effective antiviral immunity (Edelmann et al., 2004), TLR3 deficient mice inoculated with mouse cytomegalovirus had a 1000-fold augmentation of viral load in the spleen (Tabeta et al., 2004) and TLR3 deficiency in humans was shown to be associated with susceptibility to herpes simplex virus (Zhang et al., 2007).

3. Fish TLRs identified from genome and transcriptome analyses

Bony fishes are thought to have a primitive immune system and there is great scientific interest in comparing their innate and adaptive defense mechanisms with mammals. They represent approximately half of the vertebrate species and hence form the largest and most diverse group of vertebrates.

The past decade emergence of genomics research and draft genome sequences of five bony fish species led to the discovery of at least 16 TLR types in teleosts (Temperley et al., 2008) (Table 1 and Fig. 2). Key features of the fish TLRs and the factors involved in their signaling cascade have high structural similarity to the mammalian TLR system. However, the fish TLRs also exhibit very distinct features and large diversity which is likely derived from their diverse evolutionary history and the distinct environments that they occupy. The best genome-wide characterizations of fish TLR genes were done in zebrafish (*Danio rerio*) and the pufferfish (*Takifugu rubripes*) (Jault et al., 2004; Meijer et al., 2004; Oshiumi et al., 2003). A comparison of the TLRs profile of those two species reveals a core set of orthologous genes with high sequence conservation to human TLRs, but also distinct non-mammalian and even teleostei unique TLRs (Table 1 and Fig. 2). Furthermore, only some of the non-mammalian TLRs like TLR14, 21 and 22 were identified in both species, while TLR4b, 19 and 20 were only found in zebrafish and TLR5S and 23 were found in the pufferfish, but not in zebrafish. Overall, 14 distinct TLR types were identified in zebrafish by phylogenetic analysis, of which three are duplicated (TLR4b, 5 and 8), and 11 distinct types were identified in the pufferfish (Fig. 2).

Genome and gene duplications have been major contributors to the teleost's rich evolutionary history and genomic diversity, which is often ignored by fish biologists when using genomics tools in their research. The 2R genome duplication hypothesis, identifies two rounds of genome duplication in ancestral vertebrates, one immediately before and one immediately after the divergence of the lamprey lineage 500–800 million years ago (MYA) (Wolfe, 2001). The second tetraploidy event likely occurred within the subphylum Vertebrata and coincided with the development of jaws. Evidence of these phenomenons can be seen in certain gene families. Amphioxus (cephalochordates) have a single Hox gene cluster, while lampreys (jawless vertebrates) are equipped with two independent clusters. Jawed vertebrates like birds and mammals, show evidence of the second tetraploidization event by presenting three to four separate clusters of Hox genes (Ohno, 1999).

Analysis of bony fish genomes has indicated that Hox clusters and many other genome loci are present at a higher copy number than mammals, providing evidence for an ancient whole genome duplication of the teleost lineage after it split from the lobe finned lineage 325–350 MYA. For example, seven Hox gene clusters were identified in the zebrafish and other ray-fin fish genomes compared to only four Hox clusters in mammals (Amores et al., 1998, 2004). The fish specific genome duplication hypothesis or the so-called 3R hypothesis has been validated in recent years by the identifi-

Table 1
Known ligands of TLRs in fish and mammals.

TLR	Fish species	Known ligands		References
		Mammals	Fish	
TLR1	Fugu, zebrafish	Lipopeptide; Pam_3CSk_4	Unknown	Takeuchi et al. (2002), Meijer et al. (2004), and Oshiumi et al. (2003)
TLR2	Fugu, zebrafish Common carp	Lipopeptide; peptidoglycan; Pam_2CSk_4	Lipopeptides; Pam_3CSk_4	Hajjar et al. (2001), Werts et al. (2001), Meijer et al. (2004), Oshiumi et al. (2003), and Ribeiro et al. (2010)
TLR3	Fugu, zebrafish	dsRNA; polyI:C	dsRNA; polyI:C	Alexopoulou et al. (2001), Edelmann et al. (2004), Meijer et al. (2004), Oshiumi et al. (2003), and Matsuo et al. (2008)
TLR3a/b	Common carp	N/A[a]	Unknown	Kongchum et al. (2010)
TLR4	N/A	LPS	N/A	Hoshino et al. (1999)
TLR4b.a/b	Zebrafish	N/A	Unknown	Sullivan et al. (2009)
TLR5M	Fugu, rainbow trout	Flagellin	Flagellin	Hayashi et al. (2001), Tsujita et al. (2004), and Oshiumi et al. (2003)
TLR5b	Zebrafish		Unknown	Meijer et al. (2004)
TLR5S	Fugu, rainbow trout	N/A	Flagellin	Tsujita et al. (2004) and Oshiumi et al. (2003)
TLR7	Fugu, zebrafish	ssRNA; R848	Unknown	Diebold et al. (2004), Gorden et al. (2005), Meijer et al. (2004), and Oshiumi et al. (2003)
TLR7a/b	Common carp	N/A		Kongchum et al. (2010)
TLR8	Fugu	ssRNA	Unknown	Gorden et al. (2005), Oshiumi et al. (2003) and Heil et al. (2004)
TLR8a/b	Zebrafish	N/A		Jault et al. (2004) and Meijer et al. (2004)
TLR8a1/a2	Rainbow trout	N/A		Palti et al. (2010a)
TLR9	Fugu, zebrafish	CpG DNA	Unknown	Latz et al. (2004), Meijer et al. (2004), and Oshiumi et al. (2003)
TLR11	N/A	Profilin	N/A	Yarovinsky et al. (2005)
TLR14	Fugu, zebrafish	N/A	Unknown	Roach et al. (2005) and Meijer et al. (2004)
TLR19	Zebrafish	N/A	Unknown	Meijer et al. (2004)
TLR20a	Zebrafish, catfish	N/A	Unknown	Meijer et al. (2004) and Baoprasertkul et al. (2007b)
TLR21	Fugu, zebrafish	N/A	Unknown[b]	Meijer et al. (2004) and Oshiumi et al. (2003)
TLR22	Fugu, zebrafish	N/A	dsRNA; polyI:C	Jault et al. (2004), Meijer et al. (2004), Oshiumi et al. (2003), and Matsuo et al. (2008)
TLR22a/b	Atlantic salmon	N/A	Unknown	Rebl et al. (2007) and Leong et al. (2010)
TLR23	Fugu	N/A	Unknown	Roach et al. (2005)

[a] N/A – this TLR gene has not been identified in this taxa group to date.
[b] Recently, the chicken TLR21 was shown to recognize CpG DNA like the mammalian TLR9 (Keestra et al., 2010).

cation of hundreds of duplicated loci orthologous to single copy mammalian loci (Christoffels et al., 2004; Jaillon et al., 2004).

Some fish species are believed to have had an additional (4R) round of genome duplication. Among these are the catostomid fishes (Uyeno and Smith, 1972), salmonids (Allendorf and Thorgaard, 1984), common carp (Larhammar and Risinger, 1994), and goldfish. Recent molecular evidence has supported the 4R genome duplication theory in common carp and in the family Salmonidae (David et al., 2003; Koop et al., 2008; Phillips et al., 2003). In salmonids, at least 13 Hox clusters have been identified, compared to 7–8 clusters in the typical 3R teleost species (Moghadam et al., 2005a,b).

TLR7 gene duplication in genetically susceptible mouse strains was shown to be involved in the induction of systemic autoimmunity (Deane et al., 2007; Krieg, 2007). Therefore, maintaining two active copies of the same TLR gene in the genome would only make sense if each of the two forms would attain different functions to avoid "over-dosage" of TLR expression.

Paralogous, or duplicated TLR4 and TLR8 genes were identified in zebrafish (Jault et al., 2004; Meijer et al., 2004), TLR8 in rainbow trout (Palti et al., 2010a) and TLR3 and 7 in common carp (Kongchum et al., 2010). The TLR signaling transduction molecules MyD88 and TRAF6 are also duplicated in common carp (Kongchum et al., 2011). Palti et al. (2010a) concluded from a phylogenetic analysis that the two TLR8 genes in zebrafish (drTLR8a and b) were likely generated by the R3 genome duplication, while the two trout TLR8 genes are likely ohnologs (Postlethwait, 2007) of drTLR8a and were generated by the salmonid specific R4 event. Regardless of the evolutionary history of the gene duplication, caution should be applied when quantitative PCR and similar gene expression assays are designed for duplicated genes. This is particularly true in species like the salmonids or common carp that currently lack a high quality draft genome sequence and have undergone a recent (4R) genome duplication event. In our lab we have demonstrated that carefully designed quantitative gene expression assays are sensitive enough to detect differential expression of the duplicated TLR8a1 and a2 in rainbow trout (Palti et al., 2010a) and MyD88a and b or TRAF6a and b in common carp (Kongchum et al., 2011). As paralogous genes that remain active over time tend to attain new functions it is likely that they also have differential expression and quantitative PCR assays for those genes should be based on primers that can differentially amplify only one of the two paralogs. Therefore, the quantitative PCR results like those of recent studies that examined the expression of common carp TLR3 (Yang and Su, 2010) and TLR7 (Tanekhy et al., 2010) or Atlantic salmon TLR8 (Skjæveland et al., 2009) should be evaluated cautiously as the assays employed in these

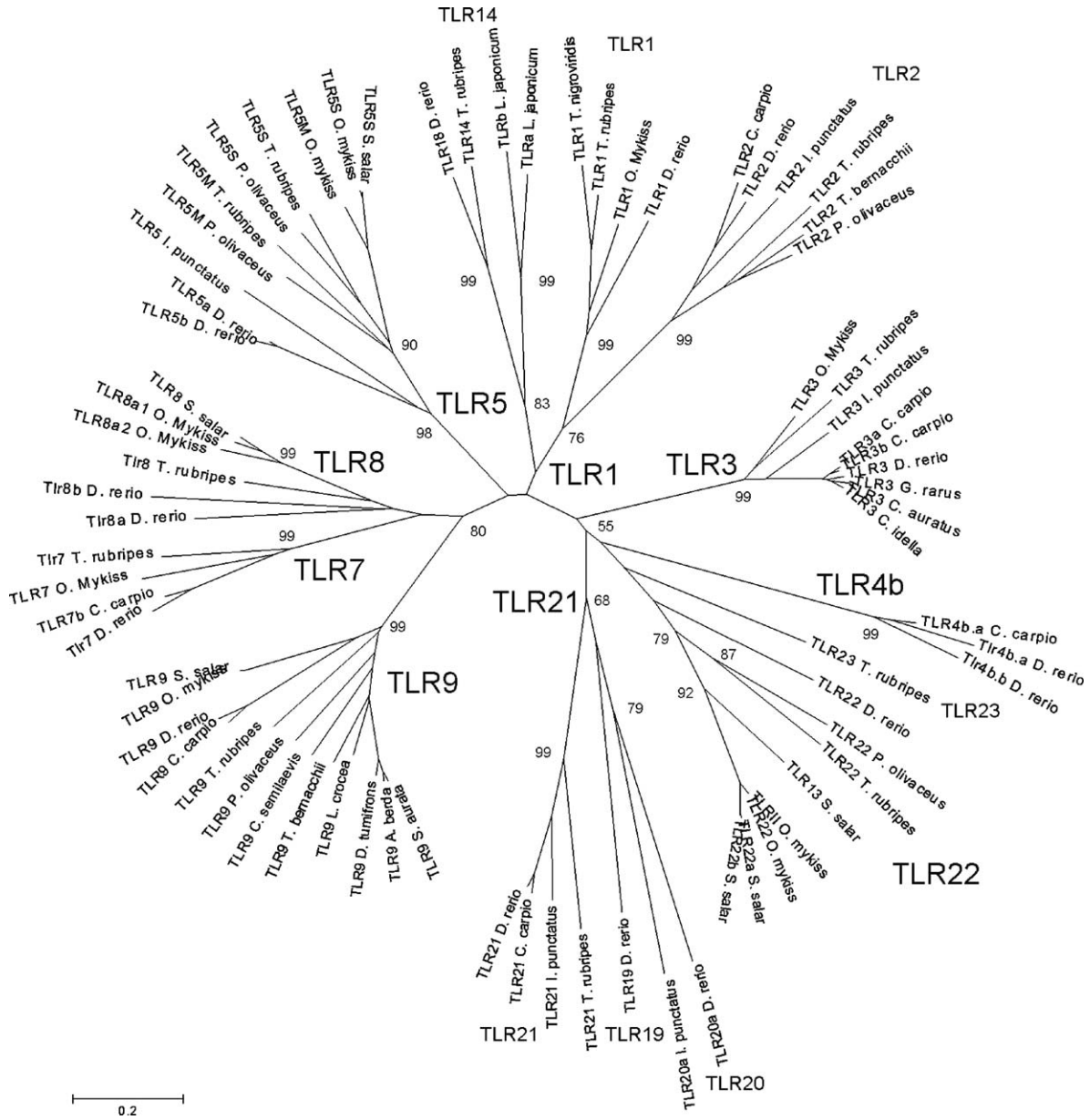

Fig. 2. Molecular phylogenetic tree of fish TLR predicted amino acid sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 20.28 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 69 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007) with 1000 bootstrap replicates. Bootstrap values of major branching points are shown as percentages. The sequences were derived from *Acanthopagrus berda* (TLR9, accession number: ABY79215), *Carassius auratus* (TLR3, ABC86865), *Ctenopharyngodon idella* (TLR3, ABI93941), *Cynoglossus semilaevis* (TLR9, ACL68661), *Cyprinus carpio* (TLR2, ACP20793; TLR3a, ABL11473; TLR3b, ADC45014; TLR4b.a, ADC45015; TLR7b, BAJ19518; TLR9, ADE20130; TLR21, ACO34812), *Danio rerio* (TLR1, NP_001124065; TLR2, NP_997977; TLR3, NP_001013287; TLR4b.a, NP_001124523; TLR4b.b, NP_997978; TLR5a, XP_001919052; TLR5b, NP_001124067; TLR7, XP_002665957; TLR8a, XP_001920594; TLR8b, XP_001340186; TLR9, NP_001124066; TLR18, NP_001082819; TLR19, XP_002664893; TLR20a, XP_001334429; TLR21, CAQ13807; TLR22, NP_001122147), *Dentex tumifrons* (TLR9, ABY79218), *Gobiocypris rarus* (TLR3, ABL11471), *Ictalurus punctatus* (TLR2, ABD17347; TLR3, ABD93872; TLR5, ABF74618; TLR20a, ABF74620; TLR21, ABF74622); *Larimichthys crocea* (TLR9, ACF60624), *Lethenteron japonicum* (TLRa, BAE47505; TLRb, BAE47506) *Oncorhynchus mykiss* (TLR1, NP_001159573; TLR3, NP_001118050; TLR5M, NP_001118216; TLR5S, NP_001117680; TLR7, ACV41797; TLR8a1, ACV41799; TLR8a2, ACV41798; TLR9, NP_001123463; TLR22, NP_001117884; TLRII, NP_001117891), *Paralichthys olivaceus* (TLR2, BAD01044; TLR5M, BAJ16366; TLR5S, BAJ16368, TLR9, BAE80691; TLR22, BAD01045), *Salmo salar* (TLR5S, NP_001117163; TLR8, NP_001155165; TLR9, NP_001117125; TLR13, NP_001133860; TLR22a, CAJ80696; TLR22b, CAR62394), *Sparus aurata* (TLR9, AAW81697), *Takifugu rubripes* (TLR1, AAW69368; TLR2, AAW69370; TLR3, AAW69373; TLR5M, AAW69374; TLR5S, AAW69378; TLR7, AAW69375; TLR8, AAW69376; TLR9, AAW69377; TLR14, AAW69369; TLR21, NP_001027751; TLR22, NP_001106664; TLR23, AAW70378), *Tetraodon nigroviridis* (TLR1, ABO15772), *Trematomus bernacchii* (TLR2, ACT64128; TLR9, ACT64130).

and similar studies did not account for the potential paralogous effect.

Sequence similarity comparisons and phylogenetic analyses of known full length predicted amino acid sequences as shown here in Fig. 2 and elsewhere in other reviews (Rebl et al., 2010; Roach et al., 2005; Temperley et al., 2008) identified eight major TLR branches in fish. The TLR1 branch which includes TLR1, TLR2 and the non-mammalian TLR14; TLR3 branch; TLR4b that shares sequence similarity, but not ligand specificity with the mammalian TLR4; TLR5 which includes membrane and fish specific soluble forms; TLR7 which includes TLR7 and 8; TLR9; the TLR21 branch which includes TLR19, 20 and 21 and the TLR22 branch (TLR22 and

23). The TLR21 and 22 branches belong to the so-called 'fish specific' TLR family and of the mammalian TLRs they share the highest sequence similarity with the murine TLR11, 12 and 13. It is important to keep in mind that while analyses of sequence similarity and genomic synteny are useful for studying the evolutionary history of fish TLRs, they are not sufficient for deducing ligand specificity and immunological properties. Known TLR ligand specificities for fish and mammals are shown in Table 1. Here below is a survey of recent literature describing what has been reported for each of the fish TLR groups.

3.1. TLR1, 2 and 14

In mammals, dimeric combinations of TLR2–TLR1 and TLR2–TLR6 recognize broad variations of bacterial peptidoglycans and lipoproteins. In fish, Ribeiro et al. (2010) found that stimulation with LTA and PGN from *S. aureus* and the synthetic triacylated lipoprotein (Pam_3CSK_4) of HEK-293 cells transected with common carp TLR2 induced MAPK-p38 phosphorylation, thus providing direct evidence for the recognition of those prototypical ligands from gram positive bacteria by the common carp TLR2. In rainbow trout, TLR1 mRNA expression in anterior kidney leukocytes was not affected by diacylated or triacylated lipoprotein treatments, or by treatment with other mammalian TLR agonists such as flagellin, poly I:C, loxoribine and R848 (Palti et al., 2010b). In zebrafish, infection with mycobacterium resulted in upregulation of TLR1, 2 and 14 mRNA eight weeks after infection (Meijer et al., 2004), and in Tetraodon, the expression level of TLR1 in the spleen of fish injected with LPS was markedly upregulated (Wu et al., 2008). In channel and blue catfish TLR2 mRNA expression in the spleen was increased one day post infection with the Gram-negative bacteria *Edwardsiella ictulari* (Baoprasertkul et al., 2007a). In Japanese flounder, TLR2 expression was upregulated in peripheral blood leukocytes after separated treatments with poly(I:C) and with peptidoglycan (Hirono et al., 2004). Purcell et al. (2006) detected moderate mRNA up-regulation of the cytokines IFN-α1 and IL-1β1 in rainbow trout kidney leukocytes stimulated with the human TLR2/6 agonist diacylated lipoprotein (Pam_2CSK_4). In the same study they found that the human TLR2/1 agonist triacylated lipoprotein (Pam_3CSK_4) had no effect on mRNA expression of a panel of six cytokines including IFN-α1 and IL-1β1.

3.2. TLR4b

The mammalian TLR4 is a central protein in the receptors' complex responding to LPS. However, LPS recognition and sensitivity in fish are fundamentally different from mammals (Rebl et al., 2010; Purcell et al., 2006; Sepulcre et al., 2007; MacKenzie et al., 2003). Most fishes lack TLR4 orthologs, with the exception of zebrafish and other cyprinidae like the rare minnow and common carp (Rebl et al., 2010; Roach et al., 2005; Temperley et al., 2008; Kongchum et al., 2010; Su et al., 2009), and all fish genomes sequenced to date lack the co-stimulatory molecules MD2 and CD14 (Sepulcre et al., 2009). Two recent studies provided compelling and direct evidence that the LPS response in zebrafish is not activated by TLR4 orthologs. Sepulcre et al. (2009) used dual-luciferase reporter assays in zebrafish embryos to study the effect of the zebrafish TLR4 orthologs on NF-κB expression. They concluded that TLR4 does not recognize LPS in fish and that the TIR domain acts as a negative regulator of MyD88-dependent signaling in zebrafish. Sullivan et al. (2009) used chimeric molecules in which portions of the zebrafish TLR4a and b proteins were fused to portions of the mouse TLR4 protein in vitro to show that the inability of the fish extracellular domains to recognize LPS, rather than changes in the TIR domains, are most likely responsible to the TLR4 lack of responsiveness to LPS. In addition, they used comprehensive syntenic and phylogenetic analyses to support the hypothesis that the zebrafish TLR4s are paralogs rather than ortologs of the human TLR4, and proposed to use the nomenclature of TLR4b, rather than TLR4, for the fish genes with sequence similarity to the mammalian TLR4s. Therefore, the appropriate names for the two zebrafish TLR4b paralogs should be TLR4b.a and TLR4b.b.

3.3. TLR5

In mammals, the membrane-bound TLR5 recognizes the flagellin protein component of bacterial flagella and is responsible for flagellin-mediated NF-κB activation. However, unlike the single receptor system in mammals, the bacterial flagellin sensing mechanism in rainbow trout was shown to be driven by membrane (TLR5M) and soluble (TLR5s) orthologs of the mammalian TLR5 in a positive feedback-loop fashion (Tsujita et al., 2004). Using chimeric fusion proteins in vitro, the two rainbow trout TLR5 forms were responsive to *Vibrio anguillarum* flagellin, and in the presence of TLR5S, flagellin-mediated NF-κB expression was increased significantly. The mRNA expression of the trout TLR5S was primarily restricted to the liver, while the TLR5M expression was found in all tissues examined. Membrane-bound and soluble forms of TLR5 were identified in other fish species as well (Oshiumi et al., 2003; Hwang et al., 2010).

3.4. TLR3

In the pufferfish fugu, Matsuo et al. (2008) showed that TLR3 is localized to the endoplasmic reticulum (ER) and recognizes relatively short dsRNA. Upon activation with its mammalian agonists polyI:C, TLR3 recruits TIR-containing adaptor molecule 1 (TICAM-1) and induces IFN expression in fish cells. Implicated evidence of endosomal localization of TLR3 in rainbow trout leukocytes based on suppressed expression of IFN2 in the presence of chloroquine, a compound known to block endosomal acidification and inhibit endosomal maturation, was reported by Palti et al. (2010a). In zebrafish, Phelan et al. (2005) used a luciferase reporter assay to show that overexpression of TLR3 in liver cells in culture can induce NF-κB expression. Several studies reported upregulation of fish TLR3s mRNA in response to infections with dsRNA viruses and/or stimulations with polyI:C in vivo and in vitro (Phelan et al., 2005; Rodriguez et al., 2005; Su et al., 2008; Chiou et al., 2007) and other studies reported IFN upregulation in rainbow trout leukocytes cell culture upon polyI:C stimulation (Palti et al., 2010a; Purcell et al., 2006). Interestingly, upregulation of TLR3 mRNA was reported in channel catfish and zebrafish upon infection with Gram-negative bacterial pathogens (Phelan et al., 2005; Bilodeau and Waldbieser, 2005; Bilodeau-Bourgeois et al., 2008), but no direct evidence for specific bacterial ligands is available.

3.5. TLR7 and 8

In mammals, TLR7 and TLR8 were shown to be activated by synthetic antiviral imidazoquinoline compounds and were implicated in recognizing single-stranded RNA, but in fish no direct evidence for ligand specificity has been shown to date. In rainbow trout anterior kidney-derived leukocytes, mRNAs of the secreted chemokines IFN1, IFN2, IL-1β1 and Il-8 were upregulated in response to treatment with R848, the human TLR7/8 agonist, but TLR7 and TLR8a1 expression levels were not affected by R848 and TLR8a2 expression was moderately down-regulated (Palti et al., 2010a). In addition, the chemokines expression in response to R848 was not suppressed in the presence of chloroquine, which may suggest that fundamental differences in TLR7/8 localization or activation exist between mammals and fish. In Atlantic salmon, the imidazoquinoline compound S-27609 induced expression of anti-viral cytokines mRNA in

liver and head kidney, possibly through the TLR7 signaling pathway (Kileng et al., 2008). In addition, Skjæveland et al. (2009) reported that Atlantic salmon TLR8 mRNA was upregulated in response to treatment with recombinant interferons in vitro, while it was not significantly affected following infection with salmon alphavirus subtype 3 in vivo.

3.6. TLR9

In mammals, TLR9 recognizes unmethylated CpG dinucleotides from viral or bacterial DNA, but in fish no direct evidence for ligand specificity has been shown to date. Several studies showed that CpG stimulation can activate antibacterial and antiviral immune responses in fishes including common carp (Tassakka ACMAR and Sakai, 2003, 2004), flounders (Lee and Kim, 2009; Liu et al., 2010), rainbow trout (Carrington and Secombes, 2007) and Atlantic salmon (Jørgensen et al., 2001a; Jørgensen et al., 2003). In zebrafish TLR9 mRNA expression was upregulated following in vivo challenge with *Mycobacterium marinum* (Meijer et al., 2004). In Atlantic salmon head kidney-derived leukocytes, TLR9 mRNA expression was moderately and significantly upregulated following treatment with CpG oligodeoxynucleotides and interferon-γ, respectively (Skjæveland et al., 2008). In Japanese flounder TLR9 has been up-regulated following cells stimulation by CpG oligodeoxynucleotides in vitro and in vivo after infection with *Edwardsiella tarda* in spleen, kidney, gills, and blood (Takano et al., 2007). Stimulation of IFN-like cytokines and IL-1β by the mammalian TLR9 agonist CpG oligodeoxynucleotide in rainbow trout anterior kidney leukocytes was shown to be suppressed by chloroquine, implicating endosomal localization of TLR9 (Jørgensen et al., 2001b).

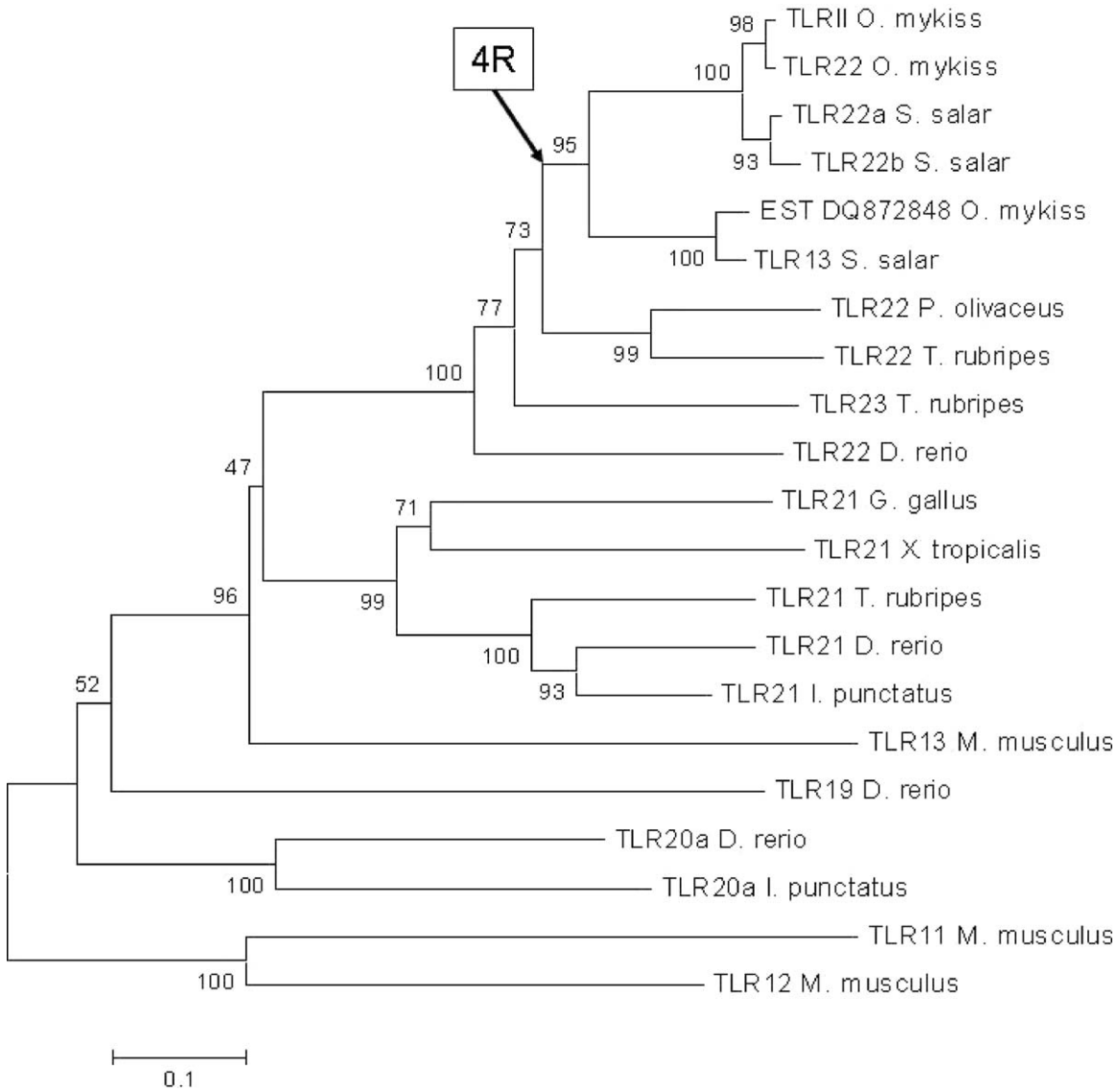

Fig. 3. Molecular phylogenetic tree of fish TLR19, 20, 21 and 22, chicken and Xenopus TLR21 and mouse TLR11, 12 and 13 predicted amino acid sequences. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 5.44 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap tests (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 234 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). The mouse (*Mus musculus*) sequences were derived from TLR11, accession number NP_991388; TLR12, NP_991392 and TLR13, NP_991389. The TLR21 sequences of the chicken (*Galus galus*) and *Xenopus tropicalis* were derived from accession numbers NP_001025729 and XP_002936443, respectively. One rainbow trout (*Oncorhynchus mykiss*) sequence derived from an EST (DQ872848) with high sequence similarity to the Atlantic salmon (*Salmo salar*) TLR13 was added to the fish sequences used in Fig. 2. An arrow is showing the predicted 4R salmonid genome duplication event as implicated from the TLR22 evolutionary history.

3.7. Non-Mammalian TLRs in Fish

The so-called 'fish-specific' family of TLRs includes TLR19, 20, 21, 22 and 23 (Rebl et al., 2010). Although they appear to branch with the murine TLR11, 12 and 13 in phylogenetic analyses (Rebl et al., 2010; Roach et al., 2005; Temperley et al., 2008), they form distinct branches (Fig. 3). As TLR21 genes were also identified in Xenopus and chicken and a TLR22 psuedogene was found in the human genome (Roach et al., 2005), it is more likely that these fish TLRs are ohnologs rather than orthologs of the murine TLR11 group and their distant ancestors became psuedogenes or were completely lost in mammalian genomes. However, better understanding of the evolutionary history of these fish TLRs can only be achieved from complete case by case genomic analyses and ligand specificity assays similar to the work completed recently for TLR4b in zebrafish (Sullivan et al., 2009). The roles of the fish specific TLRs are largely unknown. Several studies reported on stimulation experiments and RNA expression of some of those fish TLRs (Meijer et al., 2004; Hirono et al., 2004; Stafford et al., 2003; Rebl et al., 2007), but only one work to date has provided insights on ligand specificity and cellular localization. Recently, Matsuo et al. (Matsuo et al., 2008) showed that the fugu TLR22 recognizes long-sized dsRNA on the cell surface and upon activation with polyI:C it recruits TICAM-1 and induces IFN expression in fish cells. As they also showed that the fugu TLR3 is localized to the ER and recognizes relatively short dsRNA they proposed that the fugu TLR22 has a surveillance function like the human cell-surface TLR3 to alert the immune system upon extracellular infection with viral dsRNA. In chicken, TLR21 was shown to activate NF-κB in response to unmethylated CpG DNA, a ligand typically recognized by the mammalian TLR9 (Keestra et al., 2010).

Gene duplication appears to have had an important role in the evolution of the fish-specific TLR family. Six TLR20-like ORFs (TLR20a–f) were predicted in-silico from the zebrafish reference genome sequence (Meijer et al., 2004). In the pufferfishes Fugu and Tetraodon TLR22 and 23 appear to be the result of ancestral gene duplication in this lineage (Roach et al., 2005). In rainbow trout Rebl et al. (2007) identified two very similar mRNA copies of TLR22 (omTLRII and omTLR22) and more recently two genomic copies with high DNA sequence similarity to the two rainbow trout TLR22 sequences from Atlantic salmon (ssTLR22a and ssTLR22b) (Rebl et al., 2010). The phylogenetic analysis presented here (Fig. 3) suggests that those highly similar copies are not the TLR22 paralogs of the salmonid R4 event. The full length cDNA which was previously identified as ssTLR13 (Leong et al., 2010) and a rainbow trout EST that was also identified in a partial BAC sequence (DQ872850) (Palti et al., 2006) are more likely to be the R4 paralogs of the salmon and trout TLR22, but they might have also evolved through a local duplication event. The two highly similar forms identified by Rebl et al. (Rebl et al., 2007, 2010) may be the result of independent tandem duplications that occurred in Atlantic salmon and rainbow trout after the 4R event, but they may also represent allelic forms of the same locus. Genetic mapping data from my lab support the tandem duplication explanation as two rainbow trout BACs that contain partial sequences with similarity to TLR22 were mapped on chromosome 11 within 1 cM from each other and 38 cM from the BAC that harbors the trout sequence similar to the ssTLR13 (Palti et al., 2006). It is likely that the anticipated assembly of reference genome sequences for Atlantic salmon and rainbow trout will shed more light on the evolutionary history of the TLR22 paralogs in salmonids. Until then, I propose to change the name of ssTLR13 to ssTLR22b and the current ssTLR22a and b to ssTLR22a forms 1 and 2, respectively; and similarly change the names of the rainbow trout orthologs and paralogs.

In addition to the TLRs, factors involved in their signaling pathway have been studied in fish as well. Many genes similar to known factors of the mammalian TLR signaling cascade were identified in fish including recent work that illustrated the functional interchangeability of mammalian and trout factors of the TLR-signaling cascade (Rebl et al., 2011), although differences like the expression of only one TICAM gene were also observed (Rebl et al., 2010). Better functional characterization of the factors involved in this cascade in fish is needed to reveal the specific similarities and differences between fish and mammals and among the many divergent fish species.

4. Conclusions

Key features of the fish TLRs and the factors involved in their signaling cascade have high structural similarity to the mammalian TLR system. However, the fish TLRs also exhibit very distinct features and large diversity which is likely derived from their diverse evolutionary history and the distinct environments that they occupy. While the emergence of genomics research technologies has led to the discovery of the diverse fish TLRs arsenal, the exploration of the actual role of each TLR in each individual species or group of related species has just begun. Of the at least 16 TLR types identified in fish, direct evidence of ligand specificity has only been shown for TLR2, TLR3, TLR5M, TLR5S and TLR22. The main task for current and near-future fish TLRs research is to develop specificity assays to identify the ligands of all fish TLRs, which will advance comparative immunology research and will contribute to our understanding of disease resistance mechanisms in fish and the development of new adjuvants and/or more effective vaccines and therapeutics.

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